View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 39 (1991), pp. 672–683

Structural changes induced by osmotic water flow in rabbit proximal tubule

JAMES C. WILLIAMS, JR., DALE R. ABRAHAMSON, and JAMES A. SCHAFER

Department of Anatomy and Cell Biology, Medical University of South Carolina, Charleston, South Carolina; and Nephrology Research and Training Center, Departments of Physiology and Biophysics, Cell Biology and Anatomy, and Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA

Structural changes induced by osmotic water flow in rabbit proximal tubule. When a transepithelial osmotic difference was imposed in perfused proximal straight tubules (270 mOsm/kg H_2O in the lumen and 290 in the bath) in the absence of bath colloid, a severe vacuolation (appearance of lucent spaces) developed within the epithelium such that view of the lumen border was obscured within 5 ± 1 min (N = 13 tubules at 23°C). This vacuolation was less severe if the bath was hypotonic to the lumen or if the magnitude of the osmotic difference was reduced. If colloid (6% wt/vol of either bovine serum albumin or 70,000 molecular wt dextran) was included in the bathing medium, vacuolation was either not observed or was minimal, but became severe upon removal of the colloid and obscured the lumen within 6 ± 1 min (N = 8 for albumin and N = 4 for dextran at 23°C). At 38°C, vacuolation obscured the lumen within 4 ± 1 min following the removal of albumin (N = 5). ANOVA suggests that none of the times for vacuolation to occur differed. The rate of passive volume flow due to the osmotic difference was unaffected by vacuolation (0.9 ± 0.1 nl · min^{-1} · mm^{-1} with albumin to 0.8 ± 0.1 without albumin and vacuolated, N = 8 at 23°C, $P > 0.2$ using a paired t-test). Electron microscopic examination of tubules fixed after vacuolation showed lucent spaces within the cytoplasm. These results suggest that the presence of serosal colloid protected the epithelial cells from injury during rapid transepithelial water flow. The mechanism for this protective effect is not apparent, but may be related to effects of colloid in maintaining normal volume absorption in the proximal nephron.

In isolated, perfused proximal tubules from the rabbit it is not uncommon to observe a change in the appearance of the epithelium that, with brightfield optics, is characterized by a darkening of the cell layer. This change invariably starts at the perfusion end of the segment and may progress to involve the entire length of the perfused segment. The darkening of the epithelium has been called "proximal rot" [1] or "granularity" and will be referred to in this paper as "vacuolation," a term that more accurately describes the appearance of lucent spaces that accompanies the observed darkening of the epithelium. Observation of vacuolation in the epithelium has long been recognized as a sign of functional deterioration of the isolated segment, but specific causes of this change in tubule appearance have not been identified.

Received for publication May 21, 1990
and in revised form November 13, 1990
Accepted for publication November 13, 1990

© 1991 by the International Society of Nephrology

In experiments described in this paper, we induced transepithelial water flow in isolated segments of proximal tubule by imposing an osmotic difference between the perfusate and bath—with neither the perfusate nor the bath containing protein—and we found that every segment became vacuolated. However, perfused tubule segments bathed with a solution containing either bovine serum albumin or dextran (70,000 weight average molecular weight, M_w) did not undergo this change in appearance. The discovery of a reproducible way to cause vacuolation has allowed us to study this phenomenon in more detail. We have examined the time course of the development of vacuolation and the morphology of vacuolated segments using light and electron microscopy. Because the occurrence of vacuolation was related to the presence of an osmotic difference across the tubular epithelium, we also compared the rate of volume absorption driven by an osmotic difference in normal and vacuolated tubule segments and found no effect of the morphological change on the apparent osmotic water permeability of the epithelium.

Methods

Proximal straight tubules were dissected from the kidneys of New Zealand white rabbits as previously described [2], and were perfused according to the methods of Burg et al [3]. Unless otherwise indicated, the perfusate contained (in mM) KCl 5, $MgSO_4$ 0.7, $CaCl_2$ 1.5, Na-phosphates buffer 3, and sufficient NaCl (~148 mM) to bring the osmolality of the solution to 290 mOsm/kg H_2O. Albumin solution contained KCl 5, $MgSO_4$ 1, $CaCl_2$ 3, Na-phosphates buffer 3, 6 grams of purified, dialyzed bovine serum albumin per deciliter of solution, and sufficient NaCl to match the osmolality of the solution to that of the perfusate. The dextran solution was identical to the perfusate except for the addition of dextran (6%, $M_w \approx$ 70,000). Solutions were equilibrated with air and pH adjusted to 7.4. Osmolality was adjusted to 290 mOsm/kg H_2O by addition of water or NaCl, with the greatest care being taken to match the osmolalities of perfusate and bath solutions. Solutions of reduced osmolality were made by simple dilution of the above media with water. NaIsethionate solutions were made by replacement of NaCl with the sodium salt of isethionic acid. In segments perfused for measurement of volume transport, solutions were as in Schafer et al [4] as described below.

The fixative for electron microscopy was a solution like the perfusate described above except that the NaCl concentration was reduced (to ~90 mM) and glutaraldehyde was added (1.1%) to give a final osmolality of about 310 mOsm/kg H_2O. The fixative contained no colloid so as to match the colloid-free solutions bathing vacuolated segments. Before fixation the perfused segment was expelled from the distal holding pipet, fixative was flushed through the bath and the perfusion pressure was immediately reduced to zero. The perfusion pipet was withdrawn from the lumen of the segment, moved off-center, and used to push the segment out of the holding pipet. The segment was then transferred to fresh fixative in the well of a spotplate. In some cases, segments were fixed with half-strength Karnovsky's fixative [5]. After fixing for one hour at room temperature the segment was rinsed three times and stored overnight in a 0.1 M cacodylate and 3.5% sucrose buffer at 4°C. The following day the segment was post-fixed in veronal acetate buffered osmium tetroxide for 1 to 1.5 hours, stained with uranyl acetate for 1 to 1.5 hours, and embedded in agar (3% in the cacodylate buffer). A block measuring about 1 × 1 × 5 mm was cut from the agar with the tubule segment lying parallel to the long dimension. The agar block containing the segment was then dehydrated through a series of alcohols (50, 75 and 95%, each for 5 min on ice, followed by three changes of 100% at room temperature, each for 10 min), rinsed with propylene oxide (three changes, each for 5 min), and infiltrated overnight in an open container with epoxy resin diluted 1:1 with propylene oxide. The agar block was then transferred to fresh embedding medium with the tubule segment aligned along the axis of a flat mold, which was then cured for 24 hours at 60°C. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and photographed in a JEOL 100 CX electron microscope operated at 80 kV. A simple morphometric analysis of electron micrographs was undertaken where total areas of tubule cytoplasm were traced onto a Sigma-Scan digitizing tablet (Jandel Scientific) for sections taken at different lengths along the fixed segment. The number and areas of vacuoles within the cytoplasm were also recorded.

Measurement of volume absorption

In one series of experiments the rate of volume absorption driven by an imposed osmotic difference was measured. For these experiments perfusate and bath solutions identical to those described by Schafer et al [4] were used. These solutions differed from those described above in having 2 rather than 3 mM Na-phosphates buffer, the addition of 5 mM L-alanine and 8.3 mM D-glucose to the protein-containing bath, the addition of 13.3 mM urea to the protein-free perfusate to balance the alanine and glucose in the bath, and appropriate reductions in the NaCl concentration such that the osmolality of the solutions was 290 mOsm/kg H_2O. Each experiment consisted of three periods: During the first period the segments were perfused with the protein-free solution diluted to an osmolality of 270 mOsm/kg H_2O and bathed with the protein-containing solution. During the second period the bath contained either protein-containing or protein-free solution diluted to 270 mOsm/kg H_2O, so that no cryoscopic osmolality difference existed across the tubule epithelium. For the third period the bath was changed to protein-free solution at 290 mOsm/kg H_2O, establishing the same osmotic difference as in the first period but without the presence of protein in the bath. For these experiments 50 to 100 μCi of well-dialyzed, tritiated methoxy-inulin was included in each ml of perfusate as a volume marker.

Osmotic water permeability was estimated in these tubules by using a modification of a single-barrier tubule model previously described [6, 7]. Briefly, the isolated segment was modelled as a single, cylindrical membrane, and luminal flow was modelled as movement of fluid along a series of well-mixed cylindrical volumes. The steady-state concentration profiles along the length of the segment were calculated using given values for epithelial permeability, segment dimensions, perfusion rate, and media composition. To use this model for estimating osmotic water permeability, an initial guess of the permeability was made, and the steady state rate of volume absorption calculated using the length, diameter, solution compositions, and measured rates of perfusion from a single experimental period; solute permeabilities and reflection coefficients were the same as those assumed for the proximal straight tubule in reference [6]. If the calculated rate of volume absorption was not the same as that measured, a new guess of the osmotic water permeability was calculated (using Newton's method), and the cycle repeated. In this way, a value for osmotic water permeability was obtained for each perfusion period, taking into account differences in rates of perfusion and segment dimensions. The values of solute permeabilities and reflection coefficients assumed for these calculations did not influence strongly the resulting apparent osmotic water permeabilities: Changing the assumed permeabilities for Na and Cl by 50% resulted in a change of the calculated P_f by less than 2%, while a 10% change in both σ_{Na} and σ_{Cl} in one direction led to a change in the calculated P_f of about 4% in the opposite direction.

Reagents were obtained from J.T. Baker, Sigma Chemical or Electron Microscopy Sciences. Tritiated methoxy-inulin was obtained from New England Nuclear. Statistical comparisons involved the use of ANOVA or Student's *t*-test where appropriate, and differences were considered significant if P values were less than 0.05.

Results

Morphological description of the process of vacuolation

The process of vacuolation in isolated, perfused proximal tubules was observed repeatedly to begin with the appearance of a few vesicles within the tubular epithelium. With brightfield microscopy, these vesicles looked like tiny beads of oil within the otherwise translucent epithelium. As the number of these vesicles increased, the epithelium darkened and eventually became so opaque that the luminal border could not be seen. The vacuolation invariably began at the perfusion end of the segment, progressed partway toward the collection end, and eventually was accompanied by the appearance of cell fragments in the collectate that indicated degeneration of the epithelium. Typical development of vacuolation in a perfused segment of rabbit proximal straight tubule is shown in Figures 1 and 2.

Figures 3 and 4 show the electron microscopic appearance of sections of normal (Fig. 3) and vacuolated (Fig. 4) tubule segments that had been fixed using identical protocols. Normal segments showed few vacuoles, almost all of which were small and located near the apical pole of the cell (Fig. 3). In contrast,

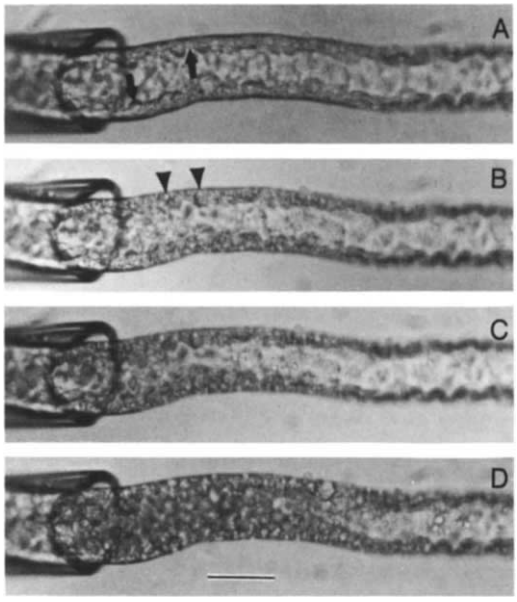

Fig. 1. *Light micrographs of isolated, perfused rabbit proximal straight tubule taken as vacuolation developed following the removal of bath protein in the presence of an osmotic difference between the perfusate and bath (perfusate 270 and bath 290 mOsm/kg H_2O).* **A.** Immediately after change to protein-free bath; note that cells are somewhat swollen but luminal boundary is clearly seen (arrows). **B.** Four minutes later; luminal border not as clearly visible as before; two vacuoles are clearly visible (arrowheads). **C.** Six minutes after bath change; epithelium has darkened at perfusion end and luminal border is scarcely visible; vacuoles have grown. **D.** Twenty minutes after bath change; vacuolation is severe and has spread somewhat down the length of the segment. Many blebs were observed flowing out of the collection end of the segment at this time. On the subjective scoring system used for Figure 7, these views were scored as 0, $1\frac{1}{2}$, $2\frac{1}{2}$, and 3 for panels A, B, C, and D, respectively. Bar = 50 μm.

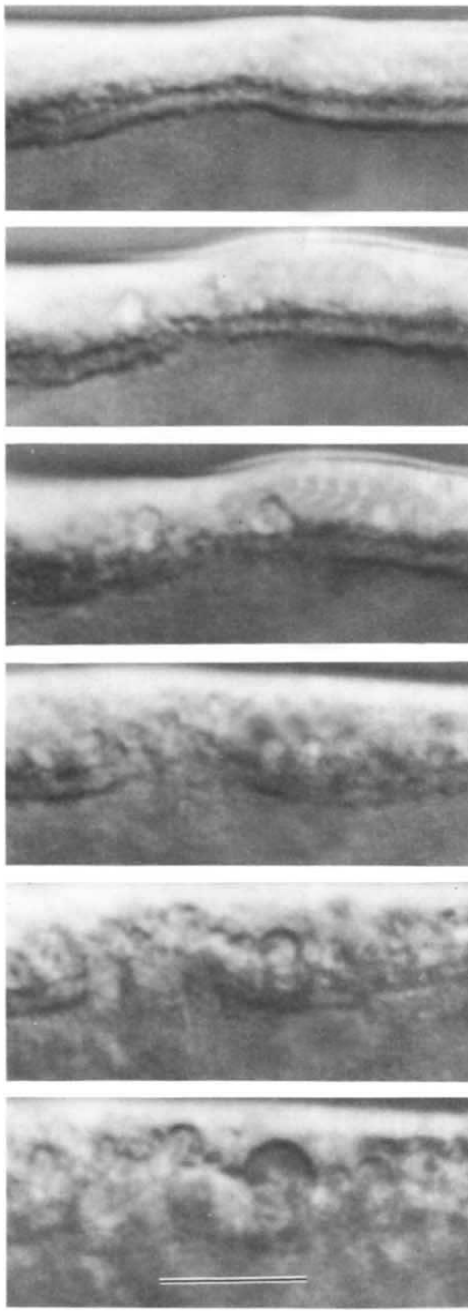

Fig. 2. *Differential interference contrast micrographs of development of vacuolation at a site about 300 μm from the perfusion end in isolated, perfused rabbit proximal straight tubule.* Osmotic difference between perfusate and bath was established as in Figure 1. Top panel shows site about 20 minutes after bath change; vacuolation had not yet spread down the length of the segment to reach this site. Other panels show, at intervals of one minute, rapid vacuolation at this site. As vacuoles develop, quality of the image is degraded. Bar = 10 μm.

the vacuolated segments showed large vacuoles located throughout the cell. Many of these vacuoles were peri-nuclear; also, few of the vacuoles were continuous with spaces that were obviously paracellular, so we believe that most of the vacuoles in these segments were intracellular. The only other repeatedly observed difference between vacuolated and normal segments was the presence of formed bodies at the luminal surface of the vacuolated tubules, structures that were probably related to blebbing of membrane from the brush border (Fig. 4).

The number and sizes of vacant spaces (vacuoles) within cells was quantitated using electron micrographs of normal and vacuolated tubules, and the results are shown in Figure 5. The number of vacant spaces was ninefold greater in vacuolated segments than in normal segments, and the total area of vacant spaces was increased twentyfold over normal. Consistent with these data, vacant spaces were measured to be bigger, on average, in the vacuolated segments compared with normal (0.86 ± 0.07 μm^2 vs. 0.45 ± 0.12 μm^2, $P < 0.05$).

We did have occasion to observe definite *paracellular* openings within the epithelium in tubule segments that were preserved with a fixative of high osmolality (half-strength Karnovsky's fixative [8, 9] with a measured osmolality of 1065 mOsm/kg H_2O), which apparently caused cell shrinkage during fixation. Figure 6 shows an electron micrograph of a normal segment fixed with half-strength Karnovsky's fixative. Note that although the overall morphology appears better than that seen with the lower osmolality fixative used for Figures 3 and 4, there is also an obvious enlargement of the paracellular spaces (Fig. 6). These spaces were visible in the light microscope, and were arranged in a roughly hexagonal pattern along the epithe-

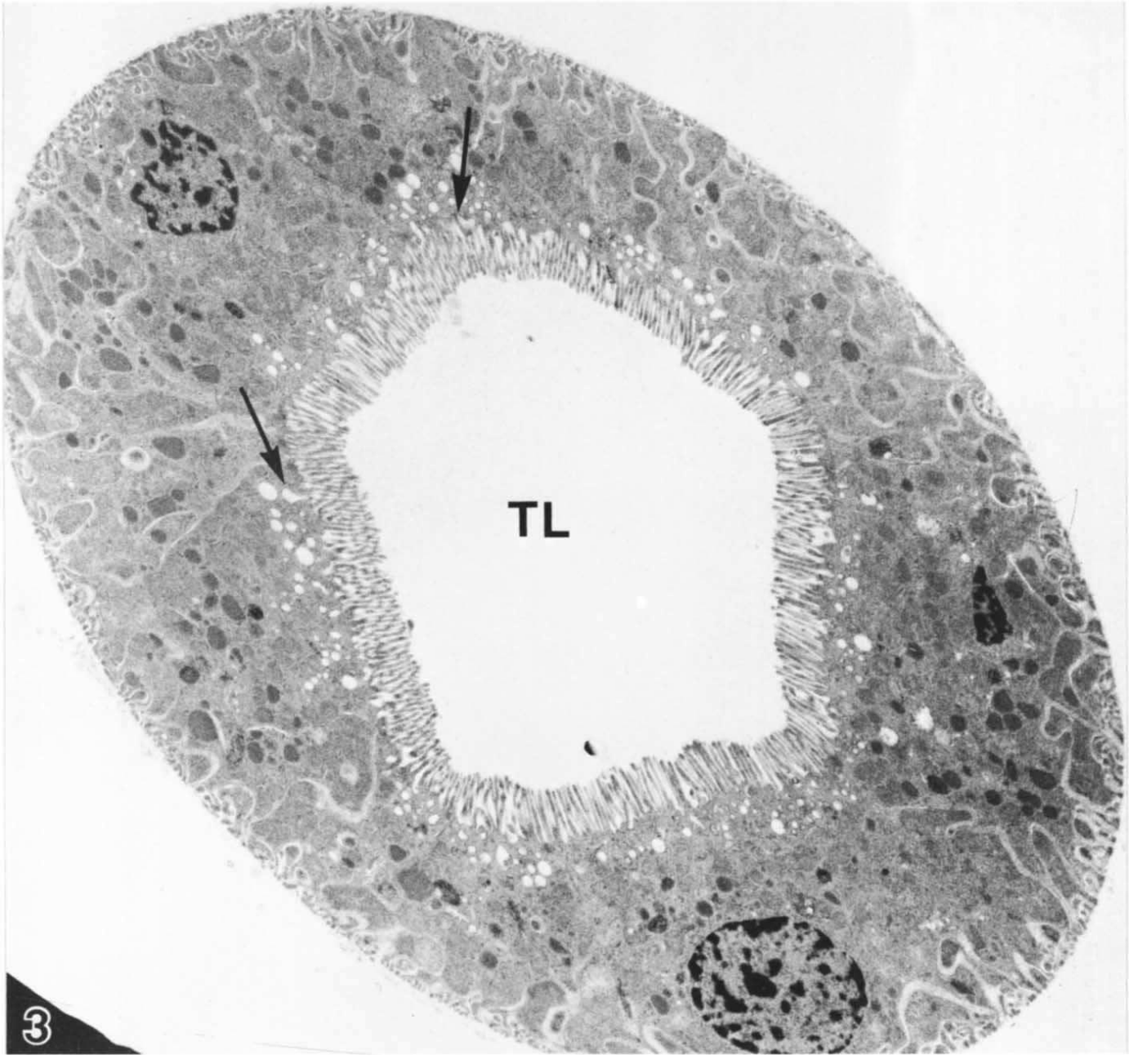

Fig. 3. *Electron micrograph of normal segment of isolated, perfused rabbit proximal straight tubule.* Segment was perfused and bathed with solution containing no colloid and an osmolality of 290 mOsm/kg H_2O. Tubular architecture similar to that in vivo is observed [12]. Invaginations of the brush border (arrows) probably represent endocytic vesicles. TL, tubular lumen. ×7500.

lium of the perfused segment. The lower-osmolality fixative used for the micrographs in Figure 3 caused no changes in the appearance of the perfused segment in the light microscope, and so was chosen for this study.

Subjective grading of degree of vacuolation

The influence of the bath-to-perfusate osmotic difference and bath colloid on the appearance of isolated, perfused proximal straight tubules is shown in Figure 7. This figure is based on a subjective scale of the appearance of the perfusion end of the segment, with a score of zero indicating an entirely normal appearance (translucent epithelium with continuous brush border), a score of one indicating the presence of a few vesicles in the epithelium, a score of two indicating that the vacuolation had progressed enough to reduce the translucency of the epithelium, and a score of three indicating that the epithelium was so opaque that the boundary of the lumen could not be seen. Even though this scale is subjective, some conclusions from the observations summarized in Figure 7 can be made with confidence. First, the imposition of an osmotic difference

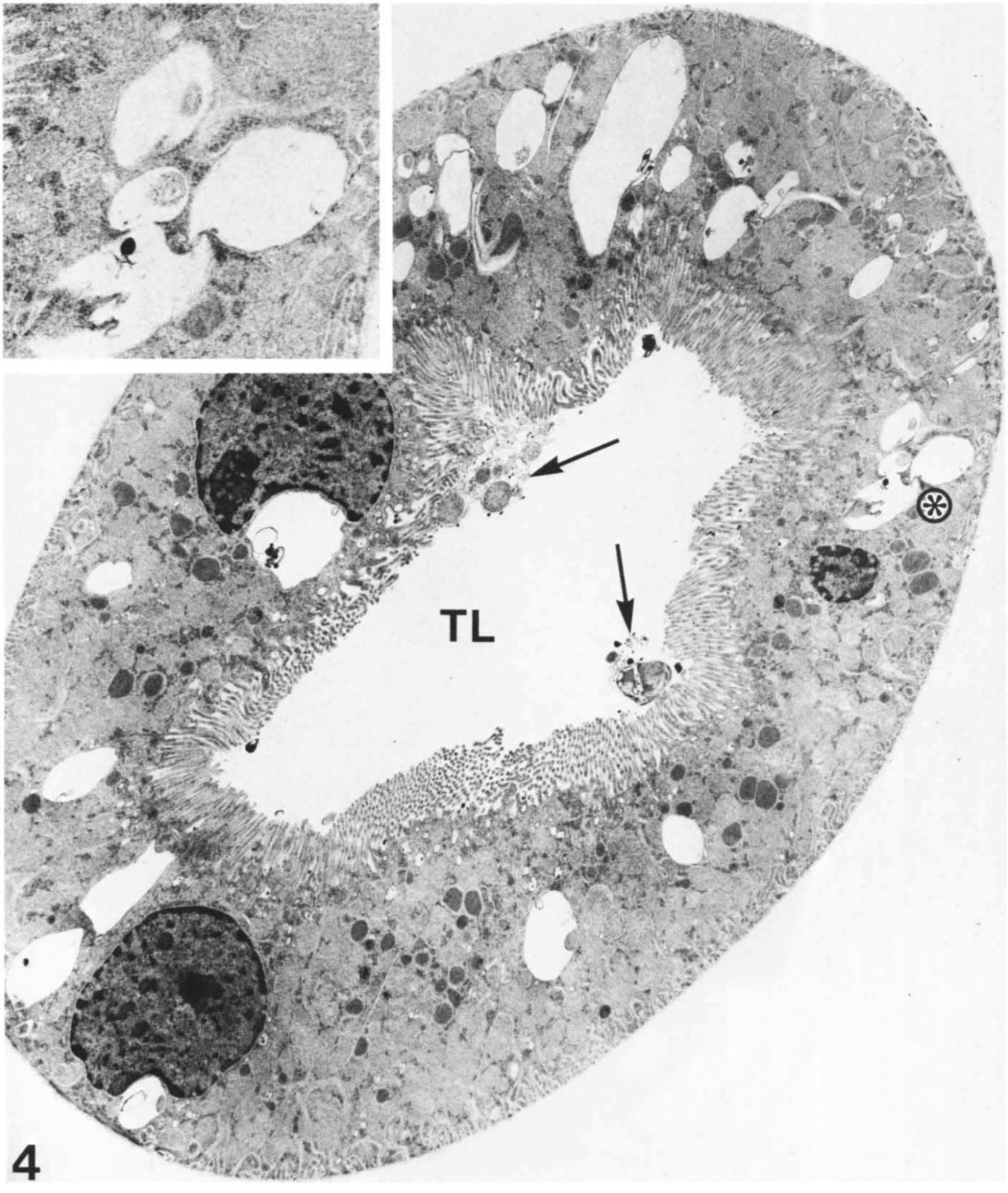

Fig. 4. *This segment was perfused with 270 and bathed with 290 mOsm/kg H_2O solutions containing no colloid.* Unlike Figure 3, numerous large vacuoles are present. Note some cell debris within tubule lumen (TL, arrows). Section was from a site 200 to 400 μm from perfusion end of segment, that is, in an area of moderate, but not severe, vacuolation. ×7500. **Inset.** Higher magnification view of area marked with *, showing vacuoles. ×19,000.

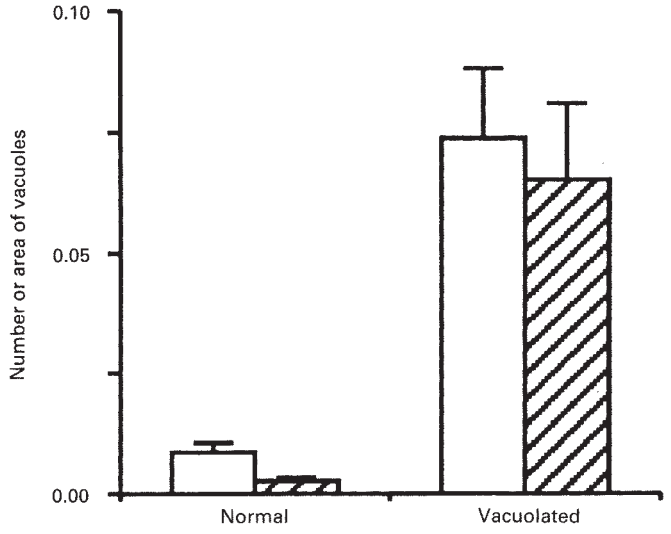

Fig. 5. *Number of vacant spaces (vacuoles) seen per square micrometer and fractional area of vacuoles in electron micrographs of proximal straight tubule segments.* Numbers for normal segments based on measurements from nine thin sections taken from three different tubules; sections were taken from sites near perfusion end of segment, middle of segment, and near the collection end. Total section area measured = 4610 μm^2. Vacuolated segments were fixed following exposure to osmotic difference (lumen 20 mOsm/kg H_2O less than bath) in the absence of bath colloid. Measurements were made on five sections from two tubules, from different sites along the length as with normal segments, with total area viewed 3060 μm^2. Both number and fractional area of vacant spaces were greater in vacuolated segments ($P < 0.001$).

between the lumen and bath led to vacuolation, and this vacuolation was generally worse when the difference was oriented with the bath hypertonic to the lumen and when the difference was 20 rather than 10 mOsm/kg H_2O. Of the 41 tubules observed with the perfusate 20 mOsm/kg H_2O less than the bath, 32 showed extreme vacuolation with the luminal border obscured, while only two of the ten tubules observed with the perfusate 10 mOsm/kg H_2O less than the bath showed this extreme vacuolation. Similarly, when the osmotic difference was reversed, none of the seven tubules observed showed extreme vacuolation. Whether a permeant salt (NaCl) or an impermeant salt (NaIsethionate) made up the bulk of the osmolality of the solutions, the responses were not significantly different. When colloid was present in the bath medium the scores for visual appearance were low, even when an osmotic difference was present across the epithelium. Vacuolation was sometimes reversible, but this maneuver was attempted in only a few cases, and never after epithelial degeneration (indicated by cell fragments emerging from the collection end of the segment) was noticed.

The time for severe vacuolation (a score of 3) to develop in perfused segments, following the imposition of an osmotic difference or after removal of bath colloid in the presence of an osmotic difference, was measured for several conditions, and the data are shown in Figure 8. The minimum time for vacuolation to become severe enough to obscure the view of the lumen was less than one minute, while the maximum time was almost 20 minutes. There was no significant difference in the time for development of the vacuolation whether no colloid was present and the bath or perfusate were changed to introduce a transepithelial osmotic difference, or whether the osmotic difference was imposed in the presence of serosal colloid and the colloid subsequently removed from the bath. Likewise, there was no significant effect on the time for development of vacuolation when dextran was used instead of albumin as the bath colloid, or when the temperature was 38°C rather than room temperature.

Volume absorption in vacuolated and non-vacuolated segments

In one series of experiments, the rate of volume absorption driven by an osmotic difference (lumen 20 mOsm/kg H_2O less than bath) was measured in segments first exposed to the osmotic difference with albumin in the bath, then to no osmotic difference with or without protein, and finally again to the osmotic difference with a protein-free bath (Fig. 9). In all cases, severe vacuolation developed during the last experimental period (Fig. 9B), but there was no significant difference between the rates of volume movement (Fig. 9A) measured in segments with little or no vacuolation (with colloid in the bath) and in segments showing severe vacuolation (those without colloid in the bath). The development of vacuolation in the perfused segments during the third period in these experiments led to a significant decrease in the rate of perfusion (Fig. 9C) due to narrowing of the lumen of the perfused segment as a consequence of cell swelling. The rate of perfusion was taken into account when osmotic water permeability was calculated (**Methods**), and the results suggest that there was no difference in apparent osmotic water permeability between periods one and three (Fig. 9D).

The ideal control for this experiment—measurement of volume flux first with no protein in the bath and then with protein—was not practicable because of the epithelial degeneration that always occurred during perfusion in the presence of an osmotic difference in the absence of bath colloid. It is possible that time-dependent changes in the osmotic water permeability could have influenced the results of this experiment, but we think this unlikely. The rate of volume absorption during the first perfusion period was quite constant, as evidenced by a mean slope of volume absorption with time of 0.0015 ± 0.0033 nl/min per minute (calculated using the linear regression of the measured rates of volume absorption on the times of the measurements, with 7 to 9 measurements for each of the 8 segments). Thus, during the first period, the rate of volume absorption showed no trend toward a steady rise or fall that could have affected results at later times.

Discussion

This study found that the imposition of an osmotic difference across the epithelium of isolated, perfused, proximal straight tubules, in the absence of bathing solution colloid, caused many large vacuoles to form in the cells of the tubule. The degree of vacuolation observed depended on the magnitude and direction of the osmotic difference imposed; its development was rapid and occurred at room temperature as well as 37°C. Vacuolation of a tubule segment did not significantly affect osmotic water permeability, but was largely prevented by the presence of

Fig. 6. *Electron micrograph of proximal straight tubule fixed using half-strength Karnovsky's, a hypertonic fixative.* Note dilated spaces between the cells (arrows). ×10,000. **Inset.** Higher magnification view of area marked with *, showing swollen intercellular spaces in continuity with basolateral membranes (arrows). ×21,000.

colloid in the bathing medium. We will first discuss the nature and development of the vacuolation, then some questions about the measurements of osmotic water permeability, and finally the intriguing finding that bath colloid can prevent the development of vacuolation in spite of the presence of osmotically driven water flow across the proximal tubular epithelium.

Injury due to transepithelial water flow

The data in Figures 1 to 4 and 7 show that an osmotic difference between the perfusate and bath in an isolated, perfused proximal straight tubule, especially in the absence of colloid in the bath, can cause development of vacuoles within the cells of the epithelium. The vacuolation of the epithelium seen in these perfused segments is like morphological changes that in older pathology textbooks are referred to as "cloudy swelling" or "hydropic or vacuolar degeneration" (for example in [8, 9]). Such texts describe these conditions to result from a wide variety of cellular insults, including osmotic injury [8].

The vacuoles seen in the present study closely resemble those that develop in vivo in proximal tubules following the intraperitoneal administration of osmotic agents such as sucrose [10, 11], while they appear larger and more basally located than those seen in segments damaged by mechanical means in vitro [12]. Other electron microscopic studies of rabbit proximal tubules perfused in vitro have not reported the presence of large vacuoles [13, 14].

Because the appearance of these vacuoles is classically a sign of tubular injury, and because in the laboratory this vacuolation presaged eventual disintegration of the cells, it must be that the vacuolation seen in this study indicates that the cells affected have been seriously injured. This sign of injury appeared soon after the imposition of osmotically driven water flow, and always appeared first at the perfusion end of the segment. Volume flow across the epithelium will increase the osmolality of the luminal fluid, and thus volume flow must lead to a lessening of the osmotic difference between lumen and bath as one moves away from the perfusion end. Therefore the greatest osmotic difference, and thus the greatest rate of volume flow, must exist at the perfusion end of the segment. The degree of such a decline in water flow with length along the segment is shown in Figure 10. Thus, the initial development of vacuoles occurred at the site of maximum volume flow, which is consis-

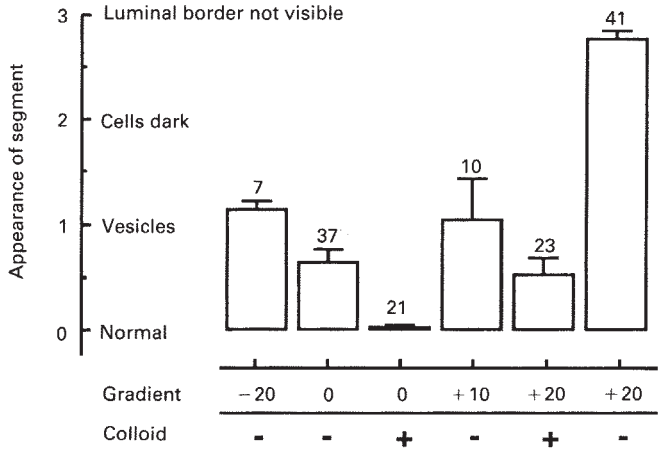

Fig. 7. *Results of subjective grading of degree of vacuolation in isolated, perfused segments of rabbit proximal straight tubule.* A positive osmotic gradient indicates number of mOsm/kg H_2O of bath greater than perfusate; presence of colloid in the bath means 6% albumin or dextran. Grading criteria were as follows: 0 if appearance was normal; 1 if vesicles (probably developing vacuoles) were seen but epithelium was still translucent; 2 if epithelium was no longer translucent at the perfusion end (Fig. 1C); and 3 if vacuolation was far enough developed that view of luminal border was obscured (Fig. 1D). Means with standard errors are shown; number of observations are shown above the bars. Total number of perfused segments used for observations was 41. In 21 segments the NaCl solutions described in the Methods (the same as those used for morphological work above) were used, while in 11 segments a similar solution with NaIsethionate replacing the NaCl was used; the remaining 9 segments were perfused with solutions as used by Schafer et al [4] and include the 8 segments shown in Figure 9. In the two rightmost bars, note that for the same imposed osmotic difference the presence or absence of bath colloid has a profound effect on the degree of vacuolation observed.

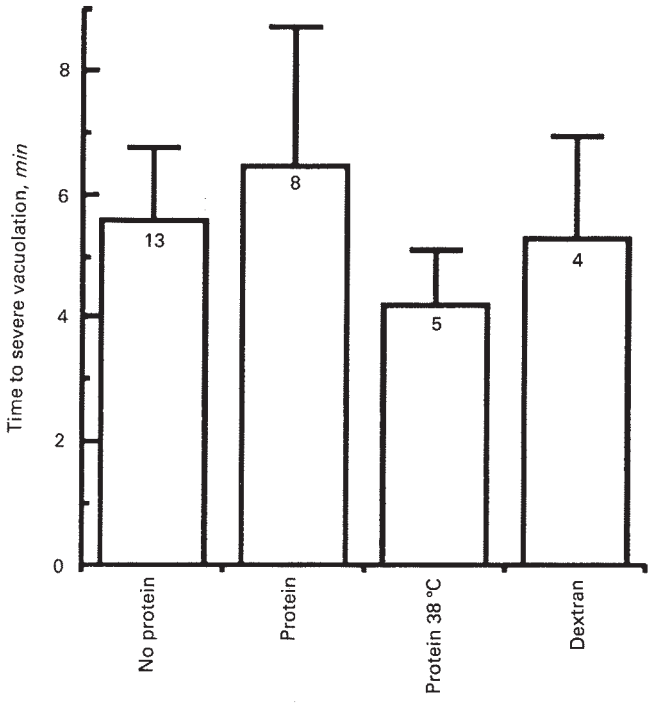

Fig. 8. *Time required for development of severe vacuolation (as in Fig. 1D) for various conditions; means with standard errors are shown.* "No protein" indicates that no colloid was contained in the media, and the time indicated was measured from the moment the bath or perfusate was changed so that the perfusate was 20 mOsm/kg H_2O lower than the bath. For the remaining three bars, the time was measured from the moment that the bath was changed to contain no colloid (protein or dextran); for these experiments the perfusate was already 20 mOsm/kg H_2O lower than the bath and remained so. Analysis of these data by ANOVA indicated no differences among the means.

tent with the volume flow being the cause of the injury leading to vacuolation.

The mechanism by which the flow of volume across the epithelium can cause injury to the cells cannot be simple dilution of the cytoplasm, as tubules perfused and bathed in low osmolality solutions did not show vacuolation (Fig. 9) and have been shown to function normally [15]. Simple osmotic shock can cause cellular vacuolation [16], but apparently the drop from 290 to 270 mOsm/kg H_2O is not sufficient for this to occur in proximal tubules. Some, or even most, of the transepithelial water flow is likely to be driven across the cells rather than through the paracellular pathway [17], and there is evidence that transcellular water flow can disrupt the normal distribution of solutes within a cell [18], but the way in which this could cause injury to a cell is not known.

Vacuolation is also observed in cortical collecting tubules during osmotically induced water flow, but no clues have yet been found to suggest the cause of this phenomenon [19, 20], which, unlike in the proximal tubule, does not lead to eventual cell degeneration. Recently, Walker et al [21] reconstructed the three-dimensional arrangement of the vacuoles found in the cortical collecting tubule from serial sections and found that most of the vacuoles were ultimately connected to the basolateral membrane by very thin (25nm) channels. It has been hypothesized that this network of "vacuoles" allows water to flow through the apical membrane and into the peritubular medium while minimizing osmotic disturbance of the basal portions of the cell [19]. However, Kirk [20], using a diffusible fluorescent marker, has established that most vacuoles in collecting tubule communicate with the peritubular medium during the process of their formation, but thereafter are entirely intracellular as judged by the persistence of the fluid-phase marker within the epithelium after the marker is removed from the bath. Thus, there may be two populations of vacuoles in the collecting tubule: one connected to the peritubular medium and one entirely intracellular.

Our morphological conclusion for the proximal tubule is simply that the primary change in segments we call vacuolated is the appearance of lucent spaces within the epithelium. While these appear to be intracellular, we have done no experiments to demonstrate this. Indeed, the conflict of data on the intracellular nature of collecting tubule vacuoles just described suggests that confirmation of the intracellular nature of vacuoles is not trivial. We are confident, however, that the presence of vacuoles in the proximal tubule following imposition of an osmotic difference is not an artifact of fixation, because lucent spaces are observed by light microscopy even before fixation. We also know that definite paracellular openings, as seen in Figure 6, appear in a hexagonal pattern along the epithelium in the light microscope and show numerous connections with

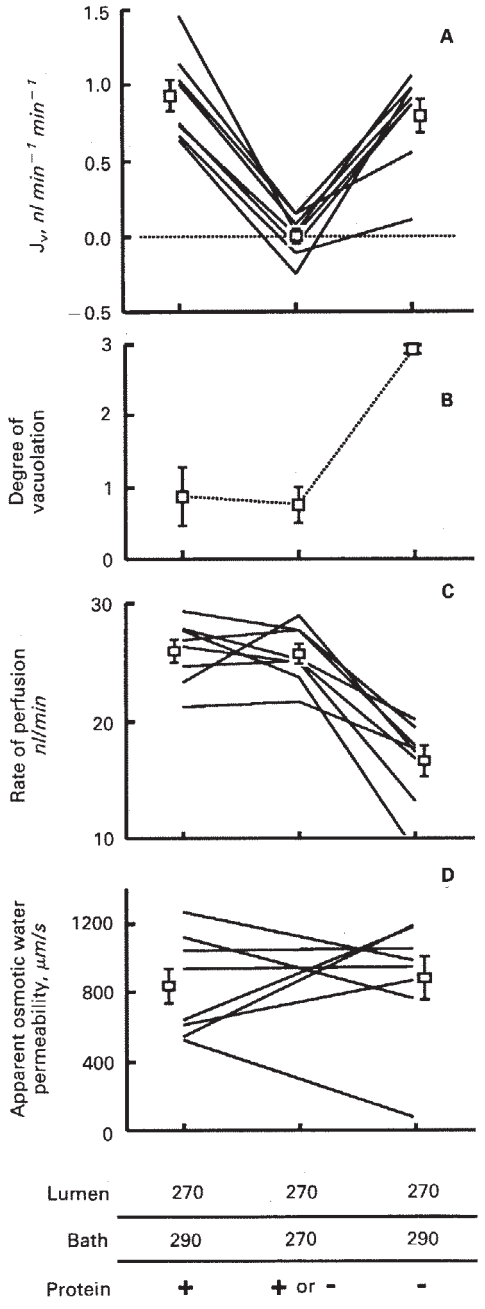

Fig. 9. *Results from series of volume absorption measurements.* Lines indicate individual tubules (N = 8), with means and standard errors shown for each perfusion period. **A.** Rates of volume absorption (J_V). **B.** Subjective scoring of vacuolation at the perfusion end of the segments (only means are shown). **C.** Perfusion rates; these tended to fall off as vacuolation increased and lumen was blocked by swelling cells. **D.** Osmotic water permeability for periods one and three as calculated using a computer model (**Methods**). Times for each perfusion period, including time for equilibration following changes (10 min each period): osmotic difference with protein, 44 ± 2 min; no osmotic difference, 32 ± 1; and osmotic difference without protein, 32 ± 4.

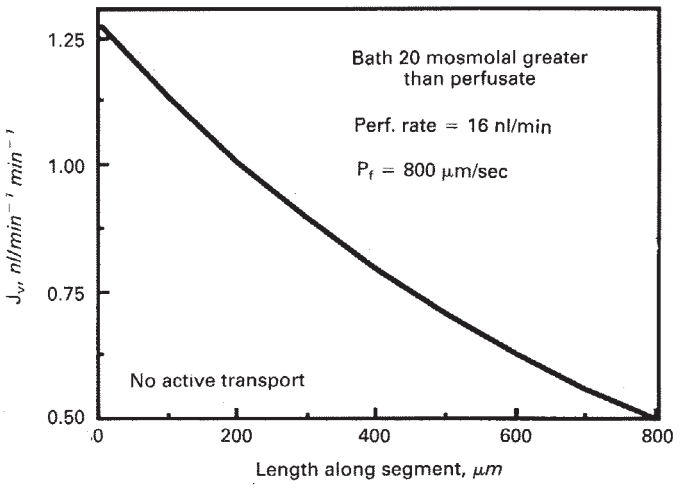

Fig. 10. *Predicted rate of volume absorption along the length of a proximal straight tubule perfused with 270 mOsm/kg H_2O and bathed with 290 mOsm/kg H_2O solutions (using computer model like that described in* ***Methods***). Solute permeabilities and reflection coefficients were assumed to be those of ref [6]. Transmural pressure was 7 mm Hg, and lumen diameter was 25 μm. Perfusion rate and osmotic water permeability (P_f) were taken to match data from period three in Figure 9. Note that rate of volume flux driven by osmotic difference falls off rapidly with distance from perfusion end; after 200 μm, rate of flux has fallen by over 20%.

paracellular spaces in the electron microscope. Similarly, dilated paracellular spaces shown by Tisher and Kokko in isolated proximal tubules are easily identified as being continuous with narrow paracellular spaces [14]. In the present study, the vacuolated segments did not show such a pattern of lucent spaces in the light microscope and showed no obvious connections with paracellular spaces, so it is likely that the vacuoles in Figure 4 are not paracellular.

Apparent osmotic water permeability

Although it is clear that no measurable change in osmotic water permeability took place during the transition of proximal tubule morphology from normal to vacuolated (Fig. 9), the magnitude of the apparent permeability in these segments is significantly smaller than that found by Schafer et al [4] using a very similar experimental design. The measurements during the first period of the series shown in Figure 9 were made using the same solutions, segments, and perfusion methods as those used by Schafer et al [4]. Figure 11 shows a comparison between the measurements made by Schafer et al and those of the present study. The lengths of perfused segments, osmotic difference, and rates of perfusion were not statistically different between the two studies, but the measured rate of volume absorption in the present study was about half of that measured in the earlier study. Using the mean values from the study of Schafer et al for volume absorption, segment length, and perfusion rate, the apparent osmotic water permeability is 2220 μm/sec, which is considerably greater than the mean in the present study of 835 μm/sec.

One unknown difference between the two studies is the hydrostatic pressure present across the tubule wall during perfusion, which was not determined in the study by Schafer et al, who used a syringe pump to drive perfusion, rather than driving perfusion by fixed pressure as in the present study. In the present study the pressure within the tubule lumen was estimated by calculating the pressure drop along the length of

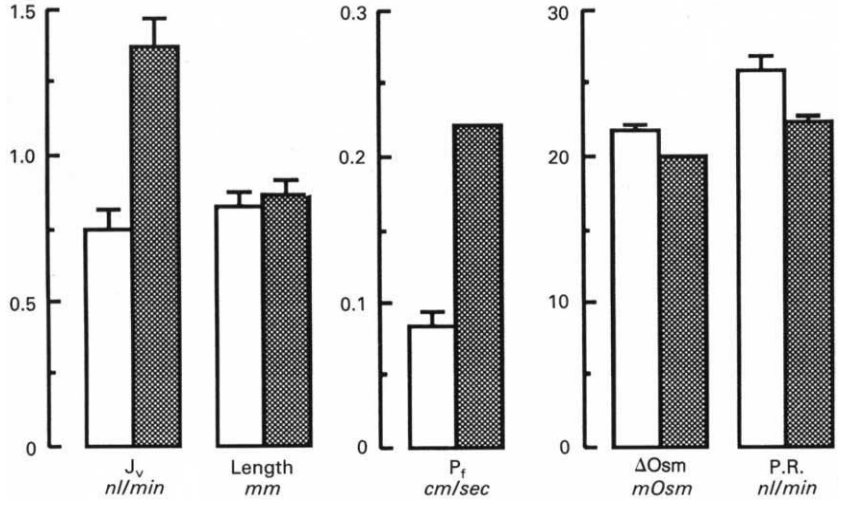

Fig. 11. *Comparison of data from the present study (□, that shown in Fig. 9) and from a series published by Schafer et al. [4] (▨); means with standard errors (SE) are shown.* All measured parameters are similar between these two series except for the rates of volume absorption (J_v) and the resulting calculations of osmotic water permeability (P_f); P_f for data from Schafer et al calculated from means so no SE shown. ΔOsm: measured osmolality difference between perfusate and bath in mOsm/kg H_2O, SE not available for Schafer et al data; P.R.: perfusion rate.

the perfusion pipet for the known rates of perfusion, using the hydraulic conductance of the perfusion pipet, which was routinely measured at the end of each experiment [6]; this estimated pressure of perfusion averaged 7 ± 2 mm Hg (about 900 pa). Working with the supposition that pressure can affect volume transport only as a direct driving force for volume movement across the epithelium, it is unlikely that a higher hydrostatic pressure in the study by Schafer et al could account for the difference in the measured rate of volume absorption between the two studies, as the rate of perfusion was not rapid enough for any reasonable pressure nearly to double the rate of volume absorption (see [6]). However, it has been proposed that perfusion pressure can modulate the response of the proximal tubule to other physical conditions [22], so that it is possible that differences in perfusion pressure could be related to the differences in the apparent osmotic water permeability between the present study and that of Schafer et al.

Recent work by Berry and Verkman suggests that the apparent osmotic water permeability is not a constant property of the proximal tubule epithelium [23]. They found that the apparent osmotic water permeability of isolated, perfused proximal convoluted tubules decreased with increasing osmotic difference, a relationship explained by the presence of a complex unstirred compartment within the epithelium. Because this apparent unstirred layer is not well-defined, it is difficult to say whether it could account for the differences in water permeability shown in Figure 11. Certainly the two studies compared in Figure 11 used very similar osmotic differences. Perhaps differences in perfusion pressure could have lead to alterations in a complex unstirred layer within the cells, and thereby to different apparent water permeabilities.

In determining the energy of activation for the water permeability, Berry and Verkman showed a sharp dependence of the permeability on temperature; for a gradient of 20 mOsm/kg H_2O they found the permeability at 39°C to be twice that at 20°C. Thus, a difference in temperature between the experiments measuring water permeability in the present study and that of Schafer et al [4] could explain the differences in apparent water permeability. However, this difference was not large; in the present study the measured bath temperatures averaged 23.3 ± 0.6°C (N = 8), which is not far from the 25°C used in the earlier study. Thus, unless some previously undescribed transition in membrane permeability or the nature of the unstirred layer occurs in the proximal tubule near 25°C, temperature difference cannot explain the discrepancy between these studies.

Peritubular colloid mitigates vacuolation induced by water flow

The ability of peritubular colloid to modify the function of renal proximal tubules has been studied by a number of laboratories, and has long been thought to play a role in the modulation of volume absorption in response to changes in vascular volume [24; for other refs., see 25]. The way in which peritubular colloid might affect tubular function is still not clear, although some laboratories have suggested that direct interaction of peritubular protein with receptors on the surface of the cells may be involved [26, 27]. The idea that the physical presence of a colloid may influence the function of the tubule has received little support.

In the present study we provide evidence that the presence of colloid, either protein or dextran, can inhibit vacuolation when water flow is driven across the epithelium by osmotic difference. Because these two colloids are chemically very different, we must conclude that the reduction in vacuolation was caused by the colloidal, or oncotic, nature of the solutions, and not to some direct interaction of macromolecules with cellular receptors. It may be that the presence of colloid simply improves the functional integrity of proximal tubule cells, or it may be that the colloid plays a specific role in preventing injury during transepithelial movement of water in this segment. Because we have not found a method for reproducibly inducing vacuolation other than by the imposition of an osmotic difference, we have not been able to test whether the presence of bath colloid affects the formation of vacuolation under conditions without transepithelial water flow.

There is evidence, however, that serosal colloid could aid in the movement of water across the basolateral membrane of the proximal tubule cell. Linshaw et al [28] found that the concentration of serosal albumin affected the cell volume of isolated,

non-perfused proximal straight tubules. Higher concentrations of serosal albumin resulted in smaller cell volumes, suggesting that serosal albumin could provide an osmotic force across the membrane that was distinct from the osmotic activity of smaller molecules. A model by Welling, Welling and Hill [29] relating cell shape to volume absorption in the proximal tubule also suggests that serosal colloid could directly affect volume movement across the basolateral membrane. In the absence of colloid, cell volume would be increased and, perhaps, thereby make the cell more vulnerable to injury by intracellular dilution.

The presence of colloid in the bath may abstract volume from the epithelium by oncotic action across the basement membrane [30]. However, reduction of such fluid movement across the basement membrane should increase the volume of the paracellular spaces, and we saw no evidence of this effect in our studies. Also, it is difficult to see how a decrease in volume abstraction across the tubular basement membrane could lead to cellular damage.

The uncertainty in these speculations points out the depth of our ignorance concerning the physical forces that are important for modulating volume absorption in the proximal tubule. Although it is well established that a transepithelial osmotic difference is the principal driving force for volume absorption in this segment [31–33], the ways in which other physical forces may act in vivo to modify this difference or the response of the epithelium to this difference are poorly understood. The results in the present study show that an osmotic difference across the epithelium of the proximal straight tubule can produce morphological changes within the cells, and that the development of these changes is affected by the presence of colloid in the bath. A more detailed explanation of these results will require a better understanding of the way that the cells of the proximal tubule react to transepithelial osmotic, oncotic and hydrostatic forces.

Acknowledgments

These studies were funded by National Institutes of Health grants DK 39023 to JCW, DK 34972 to DRA, and DK 25519 to JAS; a Grant-in-Aid from the American Heart Association to DRA; and additional funds provided by the Department of Anatomy and Cell Biology, Medical University of South Carolina. JCW and DRA are Established Investigators of the American Heart Association.

We thank Patricia St. John for technical assistance. Drs. C.H. Park and L.W. Welling provided details of the fixation methods used in their laboratories.

Reprint requests to James C. Williams, Jr., Department of Anatomy and Cell Biology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425-2204, USA.

References

1. Chonko AM, Irish JM, Welling DJ: Microperfusion of isolated tubules. *Meth Pharmacol* 4:221–258, 1978
2. Schafer JA, Troutman SL, Andreoli TE: Volume reabsorption, transepithelial potential differences, and ionic permeability properties in mammalian superficial proximal straight tubule. *J Gen Physiol* 64:582–607, 1974
3. Burg M, Grantham J, Abramow M, Orloff J: Preparation and study of fragments of single rabbit nephrons. *Am J Physiol* 210: 1293–1298, 1966
4. Schafer JA, Patlak CS, Troutman SL, Andreoli TE: Volume absorption in the pars recta. II. Hydraulic conductivity coefficient. *Am J Physiol* 234(*Renal Fluid Electro Physiol. 3*):F340–F348, 1978
5. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. (abstract) *J Cell Biol* 27:137a, 1965
6. Williams JC Jr, Schafer JA: A model of osmotic and hydrostatic pressure effects on volume absorption in the proximal tubule. *Am J Physiol* 253(*Renal Fluid Electrol Physiol* 22):F563–F575, 1987
7. Williams JC Jr, Schafer JA: The cortical interstitium as a site for solute polarization during tubular absorption. *Am J Physiol 254(Renal Fluid Electrol Physiol 23)*:F813–F823, 1988
8. Cameron GR: *Pathology of the Cell*. Edinburgh, Oliver and Boyd, 1952, p. 304
9. Robbins SL: *Textbook of Pathology*. Philadelphia, W.B. Saunders Company, 1957, p. 116
10. Merski JA, Myers MC: Light- and electron-microscopic evaluation of renal tubular cell vacuolation induced by administration of nitrilotriacetate or sucrose. *Fed Chem Toxic* 23:923–930, 1985
11. Trump BF, Janigan DT: The pathogenesis of cytologic vacuolation in sucrose nephrosis. *Lab Invest* 11:395–411, 1962
12. Nielsen JT, Christensen EI: Ultrastructure of isolated perfused proximal tubules from rabbit kidney. *Lab Invest* 49:400–411, 1983
13. Larsson L, Clapp WL, Park CH, Cannon JK, Tisher CC: Ultrastructural localization of acidic compartments in cells of isolated rabbit PCT. *Am J Physiol 253(Renal Fluid Electrol Physiol 22)*:F95–F103, 1987
14. Tisher CC, Kokko JP: Relationship between peritubular oncotic pressure gradients and morphology in isolated proximal tubules. *Kidney Int* 6:146–156, 1974
15. Williams JC Jr, Barfuss DW, Schafer JA: Transport of solute in proximal tubules is modified by changes in medium osmolality. *Am J Physiol 250(Renal Fluid Electrol Physiol 19)*:F246–F255, 1986
16. Wilkerson EH, Dibona DR, Schafer JA: Analysis of structural changes during hypotonic swelling in Ehrlich ascites tumor cells. *Am J Physiol* 251(Cell 20):C104–C114, 1986
17. Preisig PA, Berry CA: Evidence for transcellular osmotic water flow in rat proximal tubules. *Am J Physiol 249(Renal Fluid Electrol Physiol 18)*:F124–F131, 1985
18. Rick R, Dibona DR: Intracellular solute gradients during osmotic water flow: An electron-microprobe analysis. *J Membr Biol* 96:85–94, 1987
19. Dibona DR, Walker JT: Vasopressin-induced water flow and cellular geometry in the cortical collecting tubule, in *Vasopressin: Cellular and Integrative Functions*, edited by Cowley AW Jr, Liard J-F, Ausiello DA, New York, Raven Press, 1988, p. 81
20. Kirk KL: Origin of ADH-induced vacuoles in rabbit cortical collecting tubule. *Am J Physiol 254(Renal Fluid Electrol Physiol 23)*:F719–F733, 1988
21. Walker JT, Tietge JE, Condon JL, Dibona DR: On the transcellular pathway during vasopressin-induced osmotic water flow across the isolated, perfused cortical collecting tubule. (abstract) *J Cell Biol* 105:146a, 1987
22. Pirie SC, Potts DJ: The effect of peritubular protein upon fluid reabsorption in rabbit proximal convoluted tubules perfused in vitro. *J Physiol (Lond)* 337:429–440, 1983
23. Berry CA, Verkman AS: Osmotic gradient dependence of osmotic water permeability in rabbit proximal convoluted tubule. *J Membr Biol* 105:33–43, 1988
24. Brenner BM, Falchk KH, Keimowitz RI, Berliner RW: The relationship between peritubular capillary protein concentration and fluid reabsorption by the proximal tubule. *J Clin Invest* 48:1519–1531, 1969
25. Weinstein AM: Transport by epithelia with compliant lateral intercellular spaces: asymmetric oncotic effects across the rat proximal tubule. *Am J Physiol 247(Renal Fluid Electrol Physiol 16)*:F848–F862, 1984
26. Baum M, Berry CA: Peritubular protein modulates neutral active NaCl absorption in rabbit proximal convoluted tubule. *Am J Physiol 248(Renal Fluid Electrol Physiol 17)*:F790–F795, 1985
27. Webb DE, Edwards RM, Grantham JJ: Dependence of proximal tubule p-aminohippurate secretion on serum proteins and metabolic substrates. *Am J Physiol 251(Renal Fluid Electrol Physiol 20)*: F619–F626, 1986
28. Linshaw MA, Stapleton FB, Cuppage FE, Grantham JJ:

Effect of basement membrane and colloid osmotic pressure on renal cell volume. *Am J Physiol 233(Renal Fluid Electrol Physiol 2)*: F325–F332, 1977

29. WELLING DJ, WELLING LW, HILL JJ: Phenomenological model relating cell shape to water reabsorption in proximal nephron. *Am J Physiol 234(Renal Fluid Electrol Physiol 3)*:F308–F317, 1978
30. WILLIAMS JC JR: Volume fluxes driven by oncotic difference in isolated, perfused, renal tubular basement membrane. (abstract) *Kidney Int* 37(1):592, 1990
31. GREEN R, GIEBISCH G: Luminal hypotonicity: A driving force for fluid absorption from the proximal tubule. *Am J Physiol 246(Renal Fluid Electrol Physiol 15)*:F167–F174, 1984
32. LIU FY, COGAN MG, RECTOR FC JR: Axial heterogeneity in the rat proximal convoluted tubule. II Osmolality and osmotic water permeability. *Am J Physiol 247(Renal Fluid Electrol Physiol 16)*: F822–F826, 1984
33. SCHAFER JA: Mechanisms coupling the absorption of solutes and water in the proximal nephron. *Kidney Int* 25:708–716, 1984